

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
4 January 2001 (04.01.2001)

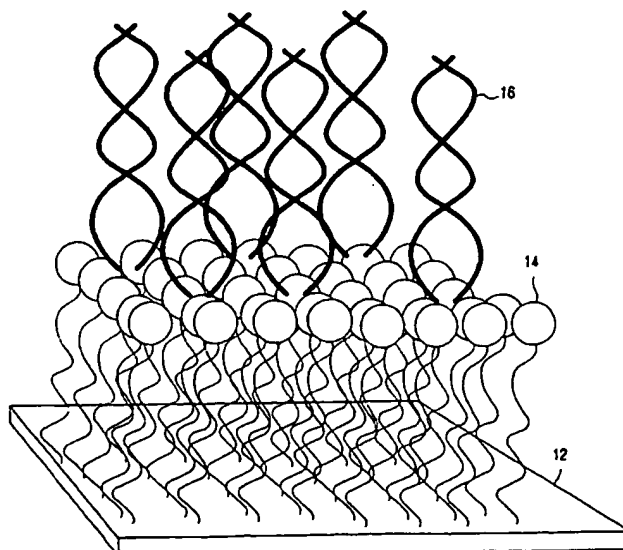
PCT

(10) International Publication Number  
**WO 01/01142 A2**

- (51) International Patent Classification<sup>7</sup>: G01N 33/543, C12Q 1/68
- (72) Inventor; and  
(75) Inventor/Applicant (for US only): CHIARYCH, Deborah [US/US]; 909 Taylor Street, Albany, CA 94706 (US).
- (21) International Application Number: PCT/US00/16894
- (22) International Filing Date: 19 June 2000 (19.06.2000)
- (74) Agents: LENTINI, David, P. et al.; Chiron Corporation, Intellectual Property - R338, P.O. Box 8097, Emeryville, CA 94662-8097 (US).
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
60/141,469 29 June 1999 (29.06.1999) US
- (63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:  
US 60/141,469 (CIP)  
Filed on 29 June 1999 (29.06.1999)
- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- (71) Applicant (for all designated States except US): CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608-2916 (US).

[Continued on next page]

(54) Title: ARRAYS OF BIOPOLYMERIC AGENTS AND METHOD FOR THEIR PRODUCTION AND USE



(57) Abstract: Arrays of biopolymeric binding agents, as well as methods for their fabrication and use, are provided. The subject arrays are characterized having at least two non-modified biopolymeric binding agents, e.g. proteins, nucleic acids, etc., bound to the hydrophilic surface of a spacer layer present a planar surface of a solid support, where the spacer layer at least includes a self-assembled monolayer. The subject arrays find use in a variety of different binding assay applications. Also provided are kits including the subject arrays.

WO 01/01142 A2

WO 01/01142 A2



**Published:**

— Without international search report and to be republished upon receipt of that report.

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

## ARRAYS OF BIOPOLYMERIC AGENTS AND METHOD FOR THEIR PRODUCTION AND USE

### TECHNICAL FIELD

5       The present invention relates to chemical synthesis and the capture and  
presentation of molecules for analysis. More particularly, the present invention relates  
to the creation of microarrays of polymeric compounds. The present invention has  
applications in the fields of organic chemistry, bioorganic chemistry, medicinal  
chemistry, genetics, genomics, proteomics, molecular biology, and analytical  
10 chemistry.

### BACKGROUND OF THE INVENTION

Microarrays of binding agents or ligands, e.g. oligonucleotides, peptides,  
proteins, etc., stably associated with the surface of a solid support have become an  
15 increasingly important tool in the biotechnology industry and related fields. These  
binding agent microarrays, in which a plurality of binding agents or ligands are stably  
associated with the surface of a solid support in the form of an array or pattern, find  
use in a variety of applications, including drug screening, oligonucleotide sequencing,  
gene expression analysis, and the like. Often in such microarrays, a complimentary  
20 binding partner docks onto the surface of the array by interaction with a binding agent  
displayed on the array surface.

One type of microarray that is currently employed in a wide variety of different  
applications, (and has potential for use in an even greater number of applications) is  
the nucleic acid array. A variety of different types of nucleic acid arrays have been  
25 developed, where differences between nucleic acid array formats include: the nature of  
the nucleic acid bound to the solid support surface; the nature of the bond between the  
nucleic acid and the support surface; the nature of the solid support; etc. Often, the  
arrays are analyzed using the emission or reflection/absorption of radiation, either  
from the materials bound to the array or from an external source.

Despite the wide variety of different types of nucleic acid arrays that have been developed to date, there is room for improvement. Problems encountered in currently available nucleic acid microarrays include: intra-array heterogeneity, irreproducibility between different arrays, high background noise, speckling, dark background, low  
5 sensitivity, high non-specific binding, irreproducibility of a given array, and the like.

As such, there is continued interest in the new array formats which overcome one or more of the above disadvantages. Of particular interest is would be the development of such an array which could be produced in a high throughput manner.

## 10 SUMMARY OF THE INVENTION

Arrays of biopolymeric binding agents associated with the surface of a solid support, as well as methods for their preparation and use, are provided. The subject arrays comprise at least two different non-modified biopolymeric binding agents, e.g. proteins, nucleic acids, etc., bound to the hydrophilic surface of a spacer layer present  
15 on the planar surface of a solid support. The spacer layer includes at least one monolayer of self-assembled molecules. The subject arrays find use in a variety of different binding assay applications, e.g. nucleic acid hybridization assays. Also provided are kits that include the subject arrays.

## 20 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A provides a three-dimensional view of a nucleic acid array according to the subject invention and Fig. 1B provides a cross-sectional view of the same array.

Fig. 2 provides the structures of representative alkylthiol compounds that find use in the subject arrays.

25 Fig. 3 provides the structure of a representative multimeric alkylthiol compound that finds use in the subject arrays.

Fig. 4 provides a comparison of two hybridization assays performed according to one embodiment of the method of the invention. The array on the left was an array having a gold surface coated glass substrate with a MUA layer. The other array was a

glass array coated with aminopropyltriethoxysilane (APTES).

### DEFINITIONS

5           The term "ligand" refers to any molecule that acts as a binding agent and is capable of participating in a specific binding reaction. Ligands of interest in the present application are biopolymers.

          The term "biopolymer" refers to an oligopeptide, polypeptide, protein, nucleic acid, ribonucleic acid, deoxyribonucleic acid, peptide nucleic acid, oligonucleotide, 10 polynucleotide, oligopeptoid, or polypeptoid, either naturally occurring or synthetically produced, or combinations thereof, including synthetic derivatives or mimetics thereof, having two or more monomeric residues. Examples of specific biopolymers of interest include: peptides, peptoids, nucleic acids, polysaccharides, etc.

          The term "peptide" as used herein refers to any compound produced by amide 15 formation between a carboxyl group of a first monomeric residue and an amino group of second monomeric residue, where each of the monomeric residues includes both an amino group and a carboxyl group, e.g. a naturally occurring or synthetic amino acid.

          The term "oligopeptide" as used herein refers to peptides with fewer than about 10 to 20 residues, *i.e.* amino acid monomeric units.

20           The term "polypeptide" as used herein refers to peptides with more than 10 to 20 residues.

          The term "protein" as used herein refers to polypeptides of specific sequence of more than about 50 residues.

          The term "nucleic acid" as used herein means a polymer made up of 25 nucleotides, e.g. deoxyribonucleotides or ribonucleotides.

          The terms "ribonucleic acid" and "RNA" as used herein means a polymer made up of ribonucleotides.

          The terms "deoxyribonucleic acid" and "DNA" as used herein mean a polymer made up of deoxyribonucleotides.

The term "oligonucleotide" as used herein denotes single stranded nucleotide multimers of from about 10 to 100 nucleotides and up to 200 nucleotides in length.

The term "polynucleotide" as used herein refers to single or double stranded polymer composed of nucleotide monomers of generally greater than 100 nucleotides  
5 in length.

The term "peptoid" as used herein refers to polymers comprising N<sup>α</sup>-substituted amides as described in U.S. Patents Nos. 5,831,005 and 5,719,049; and in co-pending U.S. Patent Applications Serial Nos. 08/340,073, 08/836,167, 08/920,205, 08/126,539, 08/277,228, 08/454,511, 08/485,106, 09/132,828; and PCT Publications  
10 Serial Nos. 96/15143 and 93/09117, each of which is incorporated herein by reference in its entirety and for all purposes.

The term "peptide nucleic acid" (or "PNA") as used herein refers to nucleic acid derivatives having a peptide or peptide-like backbone. Examples of PNAs can be found in U.S. Patents Nos. 5,831,014; 5,700,922; 5,656,461; 5,629,178; and PCT  
15 Publications Serial Nos. WO 92/20702, WO 92/20703, and WO 93/12129 each of which is incorporated herein by reference in its entirety and for all purposes.

The term "non-modified" when used to described the biopolymer binding agents of the subject invention means that the binding agent has not been modified to include a non-naturally occurring moiety, i.e. a moiety not found on a naturally  
20 occurring version of the biopolymer for the purpose of binding or otherwise associating the biopolymer with the substrate.

The term "array" as used herein refers to a composition of matter having two or more distinct binding agents stably associated with a surface as described herein.

#### DESCRIPTION OF THE SPECIFIC EMBODIMENTS

25 Arrays of biopolymeric binding agents, as well as methods for their fabrication and use, are provided. The arrays of the present invention include a substrate having a surface on which is provided a spacer layer. The spacer layer can have a substantially hydrophilic surface and includes at least one self-assembled monolayer. Bound to the



hydrophilic surface of the spacer layer, and in many instances cross-linked to the hydrophilic surface, are at least two different non-modified biopolymeric binding agents, e.g. proteins or nucleic acids. The subject arrays find use in a variety of different binding assay applications, including nucleic acid hybridization assays. Also  
5 provided are kits that include the subject arrays. In further describing the subject invention, the arrays will be discussed first in greater detail, followed by a description of representative methods for producing the subject arrays and an overview of representative applications in which the subject arrays find use.

10 Before the subject invention is described further, it is to be understood that the invention is not limited to the particular embodiments of the invention described below, as variations of the particular embodiments may be made and still fall within the scope of the appended claims. It is also to be understood that the terminology employed is for the purpose of describing particular embodiments, and is not intended  
15 to be limiting. Instead, the scope of the present invention will be established by the appended claims.

In this specification and the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Unless  
20 defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs.

25

## I. ARRAYS

As summarized above, the subject invention provides arrays of two or more biopolymeric binding agents stably associated with the surface of a solid support. The

subject arrays are characterized in one embodiment by having a spacer layer that includes at least one self-assembled monolayer on the planar surface of a base support, where the at least two biopolymeric binding agents are stably bound to the surface of the spacer layer. The biopolymeric binding agents can be associated with the surface of the spacer layer at spatially defined or discrete locations. A representation of one such array is provided in Figs. 1A and 1B. In Figs. 1A and 1B, array 10 includes a substrate 12, a spacer layer 14 and at least two different biopolymeric binding partners 16, 18 bound to the surface of the spacer layer 14, where the biopolymeric binding agents are nucleic acids 16 in Fig. 1A. Each of these components of the array is described in greater detail separately below.

A. Substrate

The solid support (Fig. 1, element 12) employed in the subject arrays may vary greatly depending on the intended use of the product to be produced. The solid support may be any suitable material for binding the spacer layer and biopolymer(s) that is also compatible with any analytical methods to be used to measure or otherwise analyze the biopolymer(s) associated with the spacer layer and/or substrate. In one embodiment, the solid support comprises an impermeable, rigid material. Suitable materials include plastics, such as polymers, e.g. polyvinylchloride, polyethylene, polystyrenes, polyacrylates, polycarbonate and copolymers thereof, e.g. vinyl chloride/propylene polymer, vinyl chloride/vinyl acetate polymer, styrenic copolymers, and the like. Suitable materials also include glasses, such as those formed from quartz, or silicon; reflective materials, e.g. aluminum; and metals, e.g. gold, platinum, silver, copper, and the like, or alloys thereof.

In many embodiments of interest, the support or substrate 12 will be a composite of two or more different layers of material, where the composition includes at least a base material, e.g. as represented by element 13 in Fig. 1, and a surface coating material, as represented by element 11 in Fig. 1. For example, one embodiment of interest includes a base material 13, such as a glass, which is coated

with a metallic layer 11, e.g. gold, silver, copper or platinum. In some more particular embodiments, the metallic layer is gold or an alloy thereof. Sandwiched in between the surface metallic layer and the base material may be one or more additional layers, e.g. adhesive metal layers (such as titanium, chromium, etc.) where these elements are not shown in Fig. 1.

In those embodiments in which the surface of the base support material, such as glass, is coated with a thin layer of a metal, such as gold, silver, platinum, copper, etc., where gold is preferred in many embodiments, as mentioned above, the thickness of the metal layer will generally range from about 300 Å to about 10,000 Å, more particularly from about 750 Å to about 2000 Å, and still more particularly from about 1000 Å to about 1500 Å. The metal layer may be deposited on the substrate surface using any suitable protocol, where suitable protocols include vapor deposition, sputtering and the like, as will be known to those of skill in the art. An adhesion metal layer may be present between the metal layer and the substrate, where adhesion metals of interest include titanium, chromium, and the like. When present, the adhesion metal layer will typically range in thickness between about 5 Å and about 100 Å, usually between about 25 Å and about 75 Å and in many embodiments will be about 50 Å. In some embodiments, the above-described adhesion layer can be a molecular adhesion layer. Examples of suitable molecular adhesion layers include mercaptopropyltriethoxysilane, etc. Where the adhesion layer is a molecular adhesion layer, the thickness of the adhesion layer typically ranges from about 5 Å to about 50 Å.

The solid support material or substrate may have a variety of different configurations, depending on the intended use of the material. Thus, in the broadest sense the support or base material may be in the form of a plate, sheet, cone, tube, well, bead, nanoparticle, etc. In some embodiments, the base support material is one that has at least one substantially planar surface, e.g. as found on a plate, slide, sheet, disc, etc. In these embodiments, supports having an overall slide or plate configuration, such as a rectangular or disc configuration are employed.

In these embodiments, the length of the support will generally be at least about 1 cm and may be as great as 40 cm or more, but will usually not exceed about 30 cm and may often not exceed about 20 cm. The width of support will generally be at least about 1 cm and may be as great as 40 cm, but will usually not exceed 30 cm and will often not exceed 20 cm. The height of the support will generally range from 0.01 mm to 10 mm, depending at least in part on the material from which the rigid substrate is fabricated and the thickness of the material required to provide the requisite rigidity. Of particular interest in many embodiments are supports having the dimensions of a standard microscope slide. Where the support is a bead or nanoparticle, the diameter of the support typically ranges from about 10nm to about 1000 $\mu$ , particularly from about 10 to about 500  $\mu$ .

#### B. Spacer Layer

As shown in Fig. 1, positioned on the surface of the substrate or support 12 is the spacer layer 14. The spacer layer is characterized by having a uniform hydrophilic surface. By uniform is meant that the surface of the spacer layer includes substantially no irregularities, such as gaps, pinholes, etc. As the surface of the spacer layer is uniform, it is substantially smooth, such that the surface of the spacer layer is homogenous. The thickness of the spacer layer may vary considerably, where the thickness may range from about 5 Å to about 5000Å, particularly from about 10 Å to about 2000 Å, and more particularly from about 20 Å to about 50 Å. In at least those embodiments where the support material includes a metallic layer beneath the spacer layer, e.g. where the support material is a gold coated microscope slide as described supra, the thickness of the spacer layer is chosen such that the spacer layer is at least sufficiently thick to separate any fluorescently labeled moiety that may be present to the surface a sufficient distance from the metallic layer on the substrate surface such that significant signal quenching (i.e. signal quenching of sufficient magnitude to

effectively preclude meaningful detection of the signal) does not substantially occur. In these embodiments, the spacer layer has a thickness that is at least about 30 Å, usually at least about 50 Å and more usually at least about 100Å, where the thickness may be as great as 2000Å or greater, but generally does not exceed about 50Å and  
5 usually does not exceed about 500Å. In those embodiments where the array is employed with fluorescently labeled target, as described in greater detail below, the thickness of the spacer layer may be chosen to provide for maximum amplification of the emitted and reflected signals. See e.g. U.S. Patent No. 5,055,265, the disclosure of which is herein incorporated by reference. In such embodiments, the thickness of the  
10 spacer layer will be about  $\frac{1}{4}$  of the wavelength of the emitted light from the label. While the exact thickness of the spacer layer will vary depending on the particular label with which it is to be employed, the thickness generally ranges from about 50 Å to about 300 Å, usually from about 100 Å to about 200 Å and more usually from about 125 Å to about 150Å.

15       Spacer layer 14 includes at least one self-assembled monolayer (SAM). As such, the spacer layer may include one or more different self-assembled monolayers grafted sequentially onto each other, where when the spacer layer includes more than one self-assembled monolayer, the spacer layer typically includes no more than 6 distinct self-assembled monolayers, and generally no more than 5 self-assembled  
20 monolayers. Where a plurality of self-assembled monolayers make up the spacer layer, uppermost self-assembled monolayer will terminate in hydrophilic functional group so as to provide an exposed distal hydrophilic surface to the spacer layer that interacts with the biopolymeric binding agents. By self-assembled monolayer is meant a layer of self-organizing or self assembling molecules which organize into an ordered, semi-  
25 crystalline structure under self-assembling conditions Examples of self-assembled monolayers can be found in the Experimental section, *infra*.

The at least one self-assembled monolayer of the spacer is typically made up of organic molecules, such that the spacer layer is an organic spacer layer. Any convenient self-assembling molecules that are capable of stably attaching to the

support surface and providing a surface layer having the above-described characteristics may be employed. The nature of the self-assembling organic molecules typically depends on the nature of the support surface on which the molecules must self-assemble to provide the requisite monolayer of the spacer layer.

5           Where the surface of the support is the surface of a metal layer, of particular interest in many embodiments is the use of self-assembling alkylthiol compounds to make up the self-assembled monolayer, or at least the self-assembled monolayer that is most proximal, i.e. adjacent, to the metal layer. By "self-assembling alkylthiol" is meant an alkylthiol molecule that is capable of assembling with additional alkylthiol  
10 molecules, which may be the same or different, to produce a self-assembled monolayer of alkylthiol molecules of substantially uniform thickness on the surface of the metallic or metallic coated substrate. As the alkylthiol molecules are self-assembling, they can form quasi-crystalline or crystalline like monolayers on the surface of the metallic substrate, where the monolayers are characterized by being substantially  
15 uniform, e.g. pinhole free. The quality of the monolayer can be evaluated using a number of art known processes and criteria, including: ellipsometry, contact angle goniometry, X-ray photoelectron spectroscopy, electrochemistry, scanning tunneling microscopy, time-of-flight secondary ion mass spectrometry, and the like, as described in: Bain et al., J. Am. Chem. Soc. (1989) 111: 7155-7164, Porter et al., J. Am. Chem.  
20 Soc. (1987) 109:3559-3568 and Offord et al., Langmuir (1994) 10:883-889.

          The alkylthiol molecules that make up the spacer layer in such embodiments of the subject invention as depicted in Fig. 1 can be characterized by an alkyl domain 17, an thio moiety 15 at proximal end and a functional group 19 at distal end. The functional group at the distal end may be any group effective to allow association of a  
25 biopolymer of interest to the spacer layer, or to couple an additional spacer layer component (e.g., such as a dicarboxylic acid) where the spacer layer is made up of sequential layers of self-assembled organic molecules grafted onto each other in a composite fashion. In one embodiment, the functional group comprises an oxygen containing moiety, e.g. -COOH, -OH or a blocked derivative thereof. Other functional

groups, e.g.  $\text{NH}_2$ , etc. can be used as well. Such groups will be familiar to those having skill in the organic and surface chemistry arts.

Examples of useful alkylthiols that find use in the production of the above described spacer layers include linear compounds that have long carbon atom chains  
5 (i.e., alkyl chains having more than about 10 carbon atoms and, more particularly, between about 10 and about 50 carbon atoms, and, still more particularly, between about 10 and about 25 carbon atoms), where additional features may include the presence of one or more heteroatoms, e.g. N, O, etc., phenyl groups, etc., where these features may be appended to the chain or incorporated into the chain. The alkyl chains  
10 may be alkane, alkene, alkyne, fluorinated alkane, etc.

In certain embodiments, e.g. where the spacer layer is made up entirely of a single, relatively thin, alkyl thiol self-assembled monolayer, the alkyl thiols will generally be  $\text{C}_8$  to  $\text{C}_{20}$ , usually  $\text{C}_{10}$  to  $\text{C}_{18}$  alkylthiol molecules that terminate at one in the  $-\text{SH}$  functionality and at the other end in an oxygen containing functionality, as  
15 described above. Representative alkylthiol groups that find use include:  $\text{C}_{11}$  alkylthiols,  $\text{C}_{18}$  alkylthiols, and the like. Specific alkylthiol compounds of interest include: (a) alcohol alkylthiols, e.g. 10-mercaptodecanol, 11-mercaptoundecanol, 12-mercaptododecanol, 14-mercaptotetradecanol, 16-mercaptohexadecanol, 18-mercaptooctadecanol, etc.; (b) acid alkylthiols, e.g. 10-mercaptodecanoic acid, 11-  
20 mercaptoundecanoic acid, 12-mercaptododecanoic acid, 14-mercaptotetradecanoic acid, 16-mercaptohexadecanoic acid, 18-mercaptooctadecanoic acid, etc.; and the like, where such compounds can be readily synthesized and/or purchase from commercial sources.

In other embodiments where a thicker spacer layer is desired, one can use self-  
25 assembling "multimeric" alkyl thiols, where by multimeric alkyl thiol is meant an alkylthiol compound that is prepared as described above and at least one additional molecule that is terminated with appropriate functionalities, e.g. amino, carboxy, etc., such that the disparate molecules or monomeric units of the multimeric alkyl thiol can be bonded to each other in head-to-tail fashion, e.g. through peptide bonds. A

representative multimeric alkyl thiol compound according to the subject invention is depicted in Fig. 2. Such compounds can be tailored to incorporate functionalities in the middle of the self-assembled layer into which they become incorporate that impart beneficial properties to the spacer layer. Such compounds are characterized by having  
5 an -SH group at a first end and an oxygen containing functionality or blocked derivative thereof at a second end. In certain embodiments, multimeric alkylthiols may be fabricated which incorporated one or more of the compounds described immediately below in connection with sequentially grafted layers, e.g. polyethylene glycols ("PEGs"), polyacrylic acids, etc.

10 Alternatively, thicker spacers can be constructed using one or more additional layers of organic compounds that have been sequentially grafted onto the base self-assembled alkylthiol layer. Representative entities that may be sequentially grafted onto the base alkylthiol layer include polymers that terminate in the requisite oxygen containing functionalities, e.g. hyper branched poly(acrylic acids), polyethylene  
15 glycols, self-assembling peptoids, multilayers of organo-disulfides, Langmuir-Blodgett multi-layers, etc. Where additional layers of organic molecules are to be grafted onto a base alkylthiol layer, one or more additional layers of molecules are sequentially grafted onto the surface of the self assembled alkylthiol layer to produce the final organic layer that displays the requisite oxygen containing functional groups and has  
20 the desired thickness.

### C. Biopolymeric Binding Agents

Bound to the hydrophilic surface of the spacer layer is at least two different  
25 biopolymeric binding agents, shown as elements 18 and 16 in Fig. 1B. A variety of different types of biopolymeric agents may be bound to the surface of the spacer layer in the subject arrays. Biopolymeric agents of interest include: nucleic acids, e.g. ribonucleic acids, such as aRNA, mRNA, etc. and deoxyribonucleic acids, e.g. PCR products, etc.; peptides, e.g. polypeptides, polypeptoids (see U.S. Patent Nos.



5,877,278; 5,811,387; and 5,789,577; the disclosures of which are herein incorporated by reference), peptide nucleic acids, and proteins. Generally, the molecular weight of the biopolymeric agents is at least about 6000 daltons, usually at least about 10,000 daltons and more usually at least about 15,000 daltons, where the weight may be as  
5 great as 1,000,000 daltons or greater, but generally will not exceed about 300,000 daltons. In some embodiments, the biopolymeric agent is a nucleic acid, e.g. a PCR product.

A critical feature of the biopolymeric binding agents is that they are non-modified. By non-modified is meant that they do not comprise any ligands or moieties  
10 configured specifically to participate in associating of the binding agent to the spacer layer. For example, where the biopolymeric binding agent is a nucleic acid, the nucleic acid does not comprise any moieties or groups which both participate in the binding of the nucleic acid to the spacer layer and are not otherwise found on naturally occurring nucleic acids, such as thiol groups, amino groups and the like. Similarly, where the  
15 biopolymeric binding agent is a protein or polypeptoid, the protein or polypeptoid does not comprise any groups that participate in the binding of the binding agent to the spacer layer that are not found on naturally occurring proteins.

The biopolymeric agents are bound to the spacer layer surface in a manner such that they are substantially stably associated with the spacer layer under the conditions  
20 of use, e.g. under hybridization, washing, and/or detection conditions such as those described in greater detail infra. In the broadest sense, any kind of attractive potential that provides a suitable substantially stable association may be present. Such potentials can be physical, e.g. electrostatic, or chemical, e.g., covalent. In many embodiments, each biopolymeric agent is covalently bonded to one or more distinct oxygen  
25 containing functionalities present on the hydrophilic surface of the spacer layer, where the covalent bond results from the cross-linking of the biopolymeric agent to the surface of the spacer layer via the oxygen containing functionalities present on the support surface.

As mentioned above, the number of different types of binding agents present

on the surface of the array is at least two. By different is meant that the sequence of monomeric units between two different biopolymeric binding agents is not the same. Where the number of different biopolymeric agents present on the surface of the array is at least 2, it is typically much higher, generally being at least about 1,000 usually at least about 5,000 and more usually at least about 10,000, where the number may be as high as 5,000,000 or higher, but typically does not exceed about 100,000 and usually does not exceed about 50,000.

#### D. General Features of the Array

10

Typically, the array is characterized by having a plurality of biopolymeric agent spots on the hydrophilic spacer layer, where each spot is characterized by having one or more, usually a plurality, of identical binding agents bound to the support surface. The number of distinct spots on the surface of the array may or may not be the same as the number of different biopolymeric agents on the array, e.g. the same biopolymeric agent may be presented in two or more spots on the array surface. Depending on the nature of the binding agents, the size of the support surface, the methods of fabrication and the intended use of the array, the number of distinct spots on the array surface may vary greatly. Where the support surface has the dimensions of a standard microscope slide, the number of spots on the support surface will typically be at least about 3000, usually at least about 6000 and more usually at least about 10,000, where the number of spots may be as great as 100,000 or greater, but generally will not exceed about 75,000 and usually will not exceed about 50,000. The diameter of each spot will typically range from about 100  $\mu\text{m}$  to about 300  $\mu\text{m}$ , usually from about 200  $\mu\text{m}$  to about 300  $\mu\text{m}$ . The space between any two given spots will generally be between about 1  $\mu\text{m}$  and about 50  $\mu\text{m}$ . The density of the spots generally ranges from about 1 to 5000 spots/ $\text{cm}^2$ , usually from about 100 to 2000 spots/ $\text{cm}^2$ . Typically, the spots are arranged across the surface of the spacer layer in the form of a pattern.

The pattern may be in the form of organized rows and columns of spots, e.g. a grid of spots, across the substrate surface, a series of curvilinear rows across the substrate surface, e.g. a series of concentric circles or semi-circles of spots, and the like.

## II. METHODS

5

The arrays of the subject invention may be prepared using any convenient protocol. One protocol of interest involves the following steps: (a) procurement of a solid support having a hydrophilic spacer layer on its surface; and (b) contact of two or more different biopolymeric agents with the support surface under conditions such that the biopolymeric agents become stably associated with the support surface.

10

### A. Solid Support Fabrication

The solid support may be fabricated using any convenient methodology, which methodology will vary depending the particular nature of the solid support. The following description is drafted in terms a glass support coated with a layer of metal, e.g. gold, as representative of the process that may be employed to prepare the solid support employed in one embodiment of the subject invention.

15

To prepare a solid support of glass coated with a metal layer, the surface of the glass is coated with a thin layer of the metal, e.g. gold, silver, platinum, or copper, etc in a thickness as described above. The metal layer may be deposited on the substrate surface using any convenient protocol, where suitable protocols include vapor deposition, sputtering, and the like, and are known to those of skill in the art. See e.g. Moteshari et al., J. Am. Chem. Soc. (1998) 120:1328-1336; Bain et al., J. Am. Chem. Soc. (1989) 111:7155-7164; Lee et al. Langmuir (1998) 14:6419-6423; Folkers et al., Langmuir (1992) 8:1330-1341, etc. Where convenient, an adhesion metal layer may be present between the metal layer and the substrate, where adhesion metals of interest include titanium, chromium, and the like, deposited in a thickness as described above.

20

25

Following preparation of gold substrate, the spacer layer is deposited onto the

surface of the gold substrate. Formation of the spacer layer on the surface of the gold substrate typically includes the step of contacting the gold surface with a solution of one or more distinct alkylthiol compounds, depending on whether the self-assembled monolayer of the spacer layer is to be homogenous or heterogeneous with respect to  
5 the spacer layer. Contact of the gold substrate with the alkylthiol solution occurs under conditions sufficient for the alkylthiol compounds to self-assemble on the surface as a single monolayer and for the thiol moieties at the base of the compounds to stably associate with the metal surface.

Generally, the alkylthiol compounds will be present in a suitable solvent,  
10 typically an organic solvent, more typically an organic polar solvent, e.g. ethanol, etc. The alkylthiol solution may be homogenous with respect to the type of alkylthiol present therein or heterogeneous as described above, where the use of heterogeneous solutions of alkylthiols, i.e. solutions of two or more different types of alkylthiols, allow for greater flexibility in tailoring the properties of the functionalized surface.  
15 The alkylthiol concentration in the alkylthiol solution is generally at least about 0.1 mM, usually at least about 0.5 mM and more usually at least about 1.0 mM, where the concentration may be as high as 100 mM or higher, but generally does not exceed about 10 mM and usually does not exceed about 5 mM.

The substrate having the metal surface may be dipped in the alkylthiol solution,  
20 the alkylthiol solution may be deposited onto the surface of the substrate, or any other convenient protocol may be employed. Typically, the gold substrate is immersed into the alkylthiol solution under conditions and for a sufficient period of time for the alkylthiol molecules to assemble into a monolayer on the substrate surface. The temperature at which contact is carried out typically ranges from about 10 to 100°C,  
25 usually from about 15 to 80°C. Contact is maintained for a sufficient period of time for the self-assembled monolayer to form on the gold surface, where contact is typically maintained for at least about 20 minutes, usually at least about 4 hours and more usually at least about 16 hours, where contact may be maintained for 36 hours or

longer, but is usually does not exceed about 24 hours and more usually does not exceed about 16 hours.

Following self assembly of the initial monolayer, one or more additional monolayers may be grafted onto the initial monolayer, where the additional layer(s)  
5 are made up of molecules, e.g. alkyls, having functionalities that provide for their covalent attachment to the surface functionalities of the initially deposited monolayer, e.g. amino functionalities where the initially deposited monolayer is characterized by the presence of carboxy functionalities. Alternatively, multi-component spacers can be constructed prior to attachment to the surface.

10 The above steps result in the production of a composite substrate having a organic spacer layer present on the surface of a gold (or other metal) substrate, where the organic spacer layer has a hydrophilic surface. The hydrophilic surface of the organic spacer can be further characterized by the presence of oxygen containing functionalities, although other suitable functionalities can be used as well. As  
15 mentioned above, oxygen containing functionalities that may be displayed on the surface are functionalities that have an -OR moiety, where R may be H or a blocking group. A variety of blocking groups may be employed, so long as the blocking group is readily removed from the oxygen containing functionality during the cross-linking step of the subject methods. Blocking groups that may be part of the oxygen  
20 containing reactive functionality include: methyl, ethyl, benzyl, t-butyl, trimethylsilylethyl, phenyl, allyl, phenacyl, as well as photolabile protecting groups and the like. Examples of suitable oxygen containing functionalities are functionalities in which the -OR moiety is bonded directly to a carbon atom, where R is H or a blocking group, as described above. Generally, the oxygen containing functionality is  
25 described by the formula  $-COR(R')(R'')$ , where R' and R'' are independently H or any group capable of being attached to a carbon atom, e.g. as seen in  $-CH_2OH$ ,  $-C(CH_3)_2OH$ , etc., or are combined as an oxygen atom double-bonded to the carbon atom, i.e. as found in  $-COOH$ . In certain embodiments, R' and R'' are groups that

modulate the reactivity of the oxygen containing functionality, e.g. make it more or less reactive. Groups of interest include electron withdrawing groups, e.g. aldehydes, esters, amides, nitro, alkyl sulfate, halo, cyano, and the like; and electron donating groups, e.g. alkyl, aryl, alkoxy, aryloxy, amino, alkylamino, and the like.

5

B. Contact of the Solid Support with Biopolymeric Binding Agents

Following preparation of the substrate, as described above, having a surface displaying the requisite oxygen containing functionalities, the two or more different biopolymeric binding agents of interest that are to be bound to the surface to produce the array are contacted with the functionalized surface. By contact is meant that the binding agents are brought into proximity with the surface such that they become substantially stably attached or bound to the hydrophilic surface of the substrate layer.

15 In contacting the binding agents with the functionalized surface, any convenient means for contacting the surface with the binding agents which results in the desired pattern of binding agent spots, as described above, may be employed. As mentioned above, the term contact is used herein to refer to any method that brings the binding agent within close proximity of the functionalized support surface. Generally, 20 an aqueous solution (e.g. water, water/organic solvent (such as 50/50 water/DMSO, and the like) of the binding agent is employed during contact where the solution may comprise one or more components in addition to water and the binding agent, e.g. buffering agents, salts, and the like. Typically, contact is achieved by depositing solutions of the different binding agents onto discrete locations of the support surface, 25 such that each different type of binding agent is deposited onto its own unique location on the functionalized surface.

The binding agents may be deposited onto the support surface using any convenient means. A number of devices and protocols have been developed for depositing aqueous solutions onto precise locations of a support surface and may be

employed in the present methods. Such devices include "ink-jet" printing devices, mechanical deposition or pipetting devices and the like. See e.g. U.S. Patent Nos. 4,877,745; 5,338,688; 5,474,796; 5,449,754; 5,658,802; 5,700,637; and 5,807,552; the disclosures of which are herein incorporated by reference. Robotic devices for  
5 precisely depositing aqueous volumes onto discrete locations of a support surface, i.e. arrayers, are also commercially available from a number of vendors, including: Genetic Microsystems; Molecular Dynamics; Cartesian Technologies; Beecher Instruments; Genomic Solutions; and BioRobotics.

Following deposition of the binding agents onto the surface of the support,  
10 support surface is dried. The support surface may be dried using any convenient protocol, e.g. under a stream of air, etc.

In certain embodiments, the above steps may be sufficient to result in the binding agent being bound to the spacer layer of the substrate in a manner such that the binding agent is sufficiently stably associated with the support surface for use in  
15 subsequent binding assay procedures. For example, where sufficient electrostatic attraction is present between the binding agents and the surface of the spacer to provide the requisite stable association of the binding agents with the support surface, no further treatment is required. However, in some embodiments, further treatment is desirable in order to covalently bond the binding agents to the hydrophilic surface of  
20 the spacer layer.

Where covalent attachment of the binding agents to the support surface is desired, the binding agents may be cross-linked to the support surface. In the present methods, the binding agents are crosslinked to the surface of the spacer layer such that the binding agents become covalently bonded to one or more oxygen containing  
25 functionalities on the hydrophilic surface of the spacer layer. Crosslinking may be achieved using any convenient methodology, where crosslinking may be achieved by chemical means, thermal means, exposure to electromagnetic radiation and the like.

Where crosslinking is achieved by exposure to the electromagnetic radiation, the surface of the support having the binding agents present thereon is typically

exposed to ultraviolet light, where the wavelength of the ultraviolet light typically ranges from about 200 to 370 nm, usually from about 225 to 360 nm, and more usually from about 250 to 300 nm. Crosslinking is carried out under substantially oxygen free conditions. As such, the environment in which crosslinking is carried is typically  
5 voided of oxygen prior to exposure to ultraviolet radiation. Any convenient means of removing oxygen from the crosslinking environment may be carried out, such as purging the local environment with an inert gas, e.g. argon, nitrogen etc, for a sufficient period of time and under sufficient pressure to remove substantially all of the oxygen from the environment, e.g. for 10 to 15 minutes under pressures ranging from  
10 5 to 100 PSI. For crosslinking, the surface of the support is irradiated with UV light at energies ranging from about 0.05 – 50 J/cm<sup>2</sup>. Following irradiation, the support is generally subject to thermal treatment, e.g. the slide is baked at 80 ° C for 10 to about 60 min or longer. The above process results in an array according to the subject invention.

15

### III. METHODS OF USING THE BINDING AGENT ARRAYS OF THE SUBJECT INVENTION

The subject arrays find use in a variety of different applications in which binding events between the surface bound binding agents of the array and analyte(s) of  
20 interest in a test sample are detected. In other words, the arrays of the subject invention find use in binding assays. In such applications, the support bound binding agent generally acts as a “target” for the analyte “probe” in the test sample. The analyte probe is typically labeled, e.g. where the label may be a directly detectable label (e.g. radioactive isotope, fluorescent label, chemiluminescent label, etc.) or an indirectly  
25 detectable label (e.g. member of a signal producing system, such as a ligand for a labeled antibody, where the label may be enzymatic which converts a substrate to a chromogenic product, etc., where the labeled antibody may be a secondary labeled antibody) so that binding events may be readily detected. The particular application in



which a given ligand display array according to the subject invention finds use necessarily depends on the nature of ligand display array. For illustrative purposes, uses for nucleic acid arrays according to the subject invention, e.g. ligand display arrays in which the ligand is a nucleic acid, such as a oligonucleotide, polynucleotide, e.g. cDNA, etc., will be described in greater detail.

Nucleic acid arrays of the subject invention find use in a variety of different applications in which one is interested in detecting the occurrence of one or more binding events between probe or analyte nucleic acids and targets on the array and then relating the occurrence of the binding event(s) to the presence of a probe(s) in a sample. In such methods, the array is contacted with a sample containing the probe nucleic acids under conditions sufficient for any probe present in the sample to bind or hybridize to complementary target nucleic acids bound to the support surface of the array. Generally, the sample is a fluid sample and contact is achieved by introduction of an appropriate volume of the fluid sample onto the array surface, where introduction can be flooding the surface with the sample, deposition of the sample onto the surface, e.g. with a pipette, immersion of the entire array in the sample, and the like. In many embodiments, the solution is deposited onto the surface and then sandwiched beneath a cover slip.

The sample is typically the product of a labeled target generation procedure. Labeled targets may be generated by methods known in the art. mRNA can be labeled and used directly as a target, or converted to a labeled cDNA target. By labeled is meant that the nucleic acid targets comprise a member of a signal producing system and are thus detectable, either directly or through combined action with one or more additional members of a signal producing system. Examples of directly detectable labels include isotopic and fluorescent moieties incorporated into, usually covalently bonded to, a nucleotide monomeric unit, e.g. dNTP or monomeric unit of the primer used to generate the nucleic acid target. Isotopic moieties or labels of interest include  $^{32}\text{P}$ ,  $^{33}\text{P}$ ,  $^{35}\text{S}$ ,  $^{125}\text{I}$ , and the like. Fluorescent moieties or labels of interest include

coumarin and its derivatives, e.g. 7-amino-4-methylcoumarin, aminocoumarin, bodipy dyes, such as Bodipy FL, cascade blue, fluorescein and its derivatives, e.g. fluorescein isothiocyanate, Oregon green, rhodamine dyes, e.g. texas red, tetramethylrhodamine, eosins and erythrosins, cyanine dyes, e.g. Cy3 and Cy5, macrocyclic chelates of  
5 lanthanide ions, e.g. quantum dye□, fluorescent energy transfer dyes, such as thiazole orange-ethidium heterodimer, TOTAB, etc. Labels may also be members of a signal producing system that act in concert with one or more additional members of the same system to provide a detectable signal. Illustrative of such labels are members of a specific binding pair, such as ligands, e.g. biotin, fluorescein, digoxigenin, antigen,  
10 polyvalent cations, chelator groups and the like, where the members specifically bind to additional members of the signal producing system, where the additional members provide a detectable signal either directly or indirectly, e.g. antibody conjugated to a fluorescent moiety or an enzymatic moiety capable of converting a substrate to a chromogenic product, e.g. alkaline phosphatase conjugate antibody; and the like. Of  
15 particular interest in certain embodiments is the use of branched DNA labels. Such labels are described in U.S. Patent No. 5,124,246; 5,710,264 and 5,849,481; the disclosures of which are herein incorporated by reference.

Generally, methods for generating labeled nucleic acid targets include the use of oligonucleotide primers. Primers that may be employed include oligo dT, random  
20 primers, e.g. random hexamers, etc. A variety of different protocols may be used to generate the labeled target nucleic acids, as is known in the art, where such methods typically rely on the enzymatic generation of the labeled target using the initial primer. Labeled primers can be employed to generate the labeled target. Alternatively, label can be incorporated during first strand synthesis or subsequent synthesis, labeling or  
25 amplification steps in order to produce labeled target.

As mentioned above, following preparation of the labeled target nucleic acid, the target nucleic acid is then contacted with the array under hybridization conditions, where such conditions can be adjusted, as desired, to provide for an optimum level of specificity in view of the particular assay being performed. Suitable hybridization

conditions are well known to those of skill in the art and reviewed in Maniatis et al, *supra* and WO 95/21944, the disclosure of which is herein incorporated by reference.

Following hybridization, non-hybridized labeled nucleic acids are removed from the support surface, conveniently by washing, generating a pattern of hybridized  
5 nucleic acids on the substrate surface. A variety of wash solutions and protocols are known to those of skill in the art and may be used.

Following these standard wash procedures, in many embodiments the surface of the support is contacted with a high salt solution, e.g. by immersing the support in a high salt concentration solution. The nature of the high salt solution is such that  
10 treatment with the high salt solution makes an fluorescently labeled probe molecules on the surface of the array readily detectable in a subsequent detection step, as described in greater detail below. A representative example of a high salt solution is 5× SSC (0.75 M NaCl, .085 M Sodium Citrate). The ionic strength of the solution may vary depending on the particular nature of the buffered saline solution. For example,  
15 ionic strengths as low as 0.1M NaCl are sufficient with 0.1M NaCl in Phosphate Buffered Saline, but not as well as 5× SSC. The appropriate high salt conditions can readily be determined by those of skill in the art.

Following contact of the slides with the high salt solution, the slides are then dried in a manner sufficient to leave a uniform salt layer on the surface of the array.  
20 The slides are dried using any convenient protocol that results in the production of the uniform salt layer on the array surface, e.g. under a stream of inert gas, such as nitrogen, argon and the like.

The resultant hybridization pattern of labeled nucleic acids may be visualized or detected in a variety of ways, with the particular manner of detection being chosen  
25 based on the particular label of the target nucleic acid, where representative detection means include scintillation counting, autoradiography, fluorescence measurement, colorimetric measurement, light emission measurement, and the like.

The resultant hybridization pattern is then processed to provide information

about the sample from which the labeled target was prepared. This processing step may include comparing the hybridization pattern to one or more additional hybridization patterns derived from different sources.

The above described methods of using nucleic acid arrays according to the  
5 subject invention find use in, among other applications, differential gene expression assays. Thus, one may use the subject methods in the differential expression analysis of: (a) diseased and normal tissue, *e.g.* neoplastic and normal tissue, (b) different tissue or tissue types; (c) developmental stage; (d) response to external or internal stimulus; (e) response to treatment; and the like. The subject nucleic acid arrays therefore find  
10 use in broad scale expression screening for drug discovery, diagnostics and research, as well as studying the effect of a particular active agent on the expression pattern of genes in a particular cell, where such information can be used to reveal drug toxicity, carcinogenicity, etc., environmental monitoring, disease research and the like.

#### 15 IV. KITS

Also provided by the subject invention are kits for performing analyte binding assays using the subject arrays. Such kits according to the subject invention will at least comprise an array according to the invention, where the array may be a nucleic  
20 acid array, or peptide array. The kits may further include one or more additional reagents employed in the method for which the array is intended. Where the array is a nucleic acid array intended for use in a hybridization assay, the kit may further include primers for generating target nucleic acids, dNTPs and/or rNTPs, which may be either premixed or separate, one or more uniquely labeled dNTPs and/or rNTPs, such as  
25 biotinylated or Cy3 or Cy5 tagged dNTPs, signal producing system members, enzymes, such as reverse transcriptases, DNA polymerases, RNA polymerases, and the like, various buffer mediums, *e.g.* hybridization and washing buffers, labeled target purification reagents and components, like spin columns, etc., signal generation and detection reagents, *e.g.* streptavidin-alkaline phosphatase conjugate, chemifluorescent

or chemiluminescent substrate, branched DNAs, and the like.

V. METHODS OF DETECTING FLUORESCENTLY LABELED PROBE ON METALLIC SURFACES

5

Also provided by the subject invention are methods of detecting fluorescently labeled compounds bound to metallic surfaces, e.g. metal coated glass microscope slides, where the metallic surface are not necessarily the arrays described above. For example, the subject invention provides methods of detecting fluorescently labeled nucleic acids hybridized to complementary nucleic acids stably associated with a metallic surface, such as gold, through any type of attachment, i.e. chemical or physical attachment. Specifically, the above described process of contacting the array of the present invention with a high salt solution is applicable for other types of arrays in which a metallic support material is employed and quenching of the fluorescent signal is a problem.

15

VI. METHODS OF CROSS-LINKING BIOPOLYMERIC BINDING AGENTS TO OTHER SUPPORT SURFACES

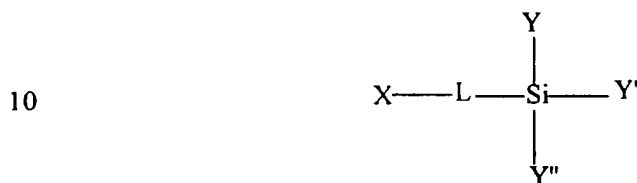
20

Also provided by the subject invention are methods of cross-linking biopolymeric binding agents to other types of array surfaces apart from those described above, i.e. arrays that lack a metal layer present beneath an organic spacer layer. In general, all that is required is that the surface of the support material be functionalized to display oxygen containing functionalities, as described above, e.g. – OH or –COOH or blocked derivatives thereof. The manner in which the support is functionalized to display these requisite functionalities varies, depending the particular nature of the support surface. The following description of the functionalization of two specific types of surfaces is representative and in no way limiting.

25

# 1. Functionalization of silicon based surfaces

For silicon based surfaces, e.g. glass, the surface of the substrate may be conveniently treated with appropriate silane compounds which are capable of introducing the desired oxygen containing functionalities onto the surface. Suitable silane compounds are generally of the formula:



In the above formula, the group X is the oxygen containing functional group, e.g. -COOP or -C(R')(R'')OP, where P is a protecting group, as described above. Y, Y' and Y'' are each independently a hydrolyzable group such as alkoxy, acyloxy, amine or chlorine, etc., or a non-hydrolyzable inert group, where at least one of Y, Y' and Y'' is a hydrolyzable group. L is a linker, where any convenient linker group may be employed, such as (CH<sub>2</sub>)<sub>n</sub>, (CH<sub>2</sub>)<sub>n</sub>-aromatic-(CH<sub>2</sub>)<sub>n</sub> or an aromatic group, etc., where n is at least 1 and usually no greater than 35. The above silane compounds can be purchased from commercial sources and/or readily synthesized by those of ordinary skill in the art. Suitable methods of using the above silane compounds to treat the surface of the support material are known to those of skill in the art, where representative protocols include contacting or treating the surface of the glass support with an aqueous/organic solvent solution of the silane compounds and then curing the treated support. The silane solution that is employed to functionalize the surface of the support may contain a single type of silane compound or two or more different types of silane compounds, e.g. silane compounds as described above that comprise X and silane compounds that lack X. By selecting appropriate mixtures of silane compounds,

greater flexibility in the nature of the surface functionalization that is provided.

Alternatively, the oxygen functionality can also be grafted onto amino-terminated silanes, such as amino-propyl triethoxysilanes which are present on the substrate surface. For example, slides (eg. aluminum or glass) modified with amino  
5 silanes (such as those that are commercially available from Amersham) are cleaned in a solution of tetrahydrofuran (THF) for 15 minutes and dried under a stream of argon. The slides are then immersed in a solution of gamma-butyrolactone, containing 1 - 10% N-methyl imidazole. The solution is stirred for 12-36 hours at 60 ° C. After the reaction  
10 is complete, the slides are rinsed in THF and dried under a stream of nitrogen. The change in hydrophobicity can be measured with a contact angle goniometer. After the reaction, the surfaces are typically 20% more hydrophilic compared to their initial amino-terminated counterpart.

## 15 2. Surfaces of a reflective material

Of interest in certain embodiments are arrays produced on reflective material surfaces. In such embodiments, the base substrate may be the reflective material or, more likely, a surface of the base material, such as glass, is coated with the reflective  
20 material. Reflective materials of interest include aluminum, gold, platinum, silver, copper and the like. In addition to alkyl thiol approach described above, the surface of the reflective material may be coated with an oxide layer to provide the requisite oxygen containing functionalities. Oxides of interest include silica oxide, Ti oxide, and the like. The thickness of this oxide layer generally ranges from about 50 to 200, usually  
25 from about 100 to 200Å. In those embodiments where the array is employed with fluorescently labeled target, as described in greater detail below, the thickness of the oxide layer is chosen to provide for maximum amplification of the emitted and reflected signals, as described above. While the exact thickness of the oxide layer will

vary depending on the particular label with which it is to be employed, the thickness generally ranges from about 50 to 300, usually from about 100 to 200 and more usually from about 125 to 150 Å. To functionalize the surface of the oxide layer, any convenient protocol may be employed, such as the silane modification protocol described above, so long as the protocol results in the display of the requisite oxygen containing functionalities on the support surface.

3. Following functionalization of the surface to display the requisite oxygen containing functional groups, biopolymeric binding agents such as nucleic acids and proteins may be cross-linked to the surface using the protocols described above. The following examples are offered by way of illustration and not by way of limitation.

## 15 EXAMPLES

### 1. Procedure for Derivatizing Gold Surfaces with Functionalized Alkyl Thiol Coatings

20 Gold-coated microscope slides were prepared by standard methods such as sputtering or vapor deposition. The slides were cleaned by immersion in chromic acid solution for 15 minutes, followed by 4 dips in pure water and 1 dip in absolute ethanol. The slides were dried under a stream of nitrogen.

25 Molecular self-assembly of the thiol solution is carried out by immersion in an 0.1 – 10 mM (typically 1 mM) solution of the appropriate thiol, typically containing a C-11 alkyl chain (see table of compounds). The typical thiol is Mercaptoundecanoic Acid (MUA), although alcohol, amine and aldehyde terminations have also been tested. The immersion time can be from 20 minutes to 24 hours, but is typically 1-3



hours. During the immersion, the thiol solution containing the slides are stirred on an orbiter at 80-100 rpm. The slides are rinsed in ethanol and dried under a stream of nitrogen.

5     2.     Procedure for grafting of subsequent layers to a Au/MUA surface

The acid-modified gold surface (Au/MUA) described in 1 above can be subsequently modified with other layered materials that contain an amine function. The amine function of the subsequent layers is covalently attached to the original acid-terminated 'base' layer of Au/MUA. Subsequent layers can consist of amino-modified  
10 poly-acrylic acids, amino acids (such as 11-amino-undecanoic acid or other chain lengths, or other amino acids), amino-modified polyethylene glycols, or poly-amido amine ("dendrimers") of various diameters. These subsequent layers are used to increase the distance between the attached DNA molecules and the hard metallic  
15 surface. It is expected that by increasing the distance between the metal (gold, platinum, silver, copper) and the fluorescent probe, greater amplification of the probe signal will result and quenching of the fluorescent signal by the metal will be substantially eliminated.

20     For the above coupling procedure, the Au/MUA surface film is activated by dipping into a solution containing 400  $\mu$ L N-methylmorpholine, 1 mL isobutyl chloroformate in 50 mL dry DMF for 10 minutes. The slides are rinsed briefly in ethyl acetate, then transferred to a solution of the appropriate amine (typically 0.5 – 2 mM). The coupling reaction is carried out for 20 minutes – 24 hours.

25

3.     Procedure for UV Cross-linking DNA to Oxygen-Containing Films on Gold Surfaces

The DNA PCR products are spotted onto the gold slides using the robotic array spotter. The humidity in the spotting chamber is adjusted to 45 – 50%. The humidity can range from 30 – 60 %. After the spots are dried, the gold slides are cross-linked in a specially designed UV cross-linking chamber equipped with an argon gas inlet. The chamber is purged for 10-15 minutes with argon at a pressure of 13 PSI. After purging the slides are irradiated with UV light at 254 nm, 0.5 J/cm<sup>2</sup>. After irradiation, the slides are baked at 80 ° C for 60 minutes.

4. Procedure for maximizing signal on Au/MUA surfaces.

After spotting, UV cross-linking and baking, the slides are “pre-hybridized” in a solution that contains all the components of the hybridization, except the probes. The solution is typically 5× SSC, 0.2% SDS, 50% formamide.

5. Probe Preparation and Hybridization

DNA probes are prepared by reverse transcription of the appropriate RNA using nucleotides that contain Cy3 or Cy5-labelled cytosines. The labeled probes are purified, concentrated, and dissolved in the appropriate hybridization solution such as 50% formamide, 5× SSC (sodium chloride / sodium citrate) , 0.1% SDS. A 25 µL aliquot of the probe solution is deposited on the slide and sandwiched underneath a cleaned coverslip. The slides are incubated for 24 hours at 42 °C. The slides are then cleaned in a solution of 1× SSC, 0.2% SDS, followed by 0.1× SSC, 0.2% SDS.

6. High Salt Solution Treatment

After these standard cleaning procedures, the gold slides are then immersed in a solution of 5× SSC (0.75 M NaCl, .085 M Sodium Citrate). The high salt solution

allows high fluorescent signals to be obtained on the Au/MUA substrate. The slides are then dried in a stream of nitrogen and scanned.

7. Fig. 4 provides a comparison of two hybridization assays performed as  
5 described above, where the array on the left was an array having a gold surface coated glass substrate with a MUA layer according to the present invention, and the other array was a glass array coated with aminopropyltriethoxysilane (APTES).

10 It is evident from the above results and discussion that the subject invention provides for a number of contributions to the field of microarrays. Specifically, the subject invention provides for the use of fluorescently labeled probes with gold surface microarrays. When used with fluorescently labeled probes, the subject arrays yield  
15 better signals, with reduced speckling, brighter background and greater sensitivity. In addition, the subject arrays are easily fabricated and are amenable to high throughput fabrication processing. As such, the subject invention provides for a significant contribution to the art.

20 All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

25

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or

**WO 01/01142**

**PCT/US00/16894**

32

scope of the appended claims.

WHAT IS CLAIMED IS:

1. An array of biopolymeric binding agents stably attached to the surface of a solid support, said array comprising:
  - 5 a substrate having a substantially planar surface;
  - a spacer layer on said planar surface, wherein said spacer layer comprises a self-assembled monolayer; and
  - at least two different non-modified biopolymeric binding agents bound to said spacer layer.
- 10 2. The array according to Claim 1, wherein said substrate is coated with a metal on said planar surface beneath said spacer layer.
3. The array according to Claim 1, wherein said spacer layer comprises a  
15 hydrophilic surface.
4. The array according to Claim 1, wherein said spacer layer comprises multiple layers of self-assembled monolayers.
- 20 5. The array according to Claim 1, wherein said non-modified biopolymeric binding agents are cross-linked to said spacer layer.
6. An array of at least two different nucleic acids stably associated with the surface of a solid support, said array comprising:
  - 25 a solid support having a planar surface coated with a metal coating layer;
  - an organic spacer layer stably associated with the surface of said metal coating layer, wherein said organic spacer layer comprises a self-assembled monolayer of alkylthiols and has a hydrophilic surface comprising oxygen containing functional groups; and

at least two different non-modified nucleic acids bound to said hydrophilic surface.

7. The array according to Claim 6, wherein said metal is gold.
8. The array according to Claim 6, wherein said substrate is glass.
9. The array according to Claim 6, wherein said oxygen containing functional groups are selected from the group consisting of:  $-C(R')(R'')OH$ ,  $-COOH$  or a blocked derivative thereof.
10. The array according to Claim 6, wherein said organic spacer layer comprises multiple self-assembled monolayers.
11. The array according to Claim 6, wherein said organic spacer layer comprises a single self-assembled monolayer.
12. The array according to Claim 11, wherein said single self-assembled monolayer comprises alkylthiol compounds that terminate in an oxygen containing functionality.
13. The array according to Claim 6, wherein said at least two different non-modified nucleic acids are cross-linked to said hydrophilic surface.
14. A method of making an array comprising at least two different biopolymeric binding agents stably associated with the surface of a solid support, said method comprising:  
placing a spacer layer on a planar surface of said solid support, wherein said

spacer layer comprises a self-assembled monolayer; and

contacting at least two different non-modified biopolymeric binding agents with the surface of said spacer layer under conditions sufficient for said non-modified biopolymeric binding agents to become bound to said spacer layer surface;

5 whereby said array is produced.

15. The method according to Claim 14, wherein said planar surface comprises a metal.

10 16. The method according to Claim 14, wherein said placing step comprises contacting said planar surface with a solution of self-assembling alkylthiols.

17. The method according to Claim 14, wherein said contacting step further comprises cross-linking said non-modified biopolymeric binding agents to said surface.

15

18. The method according to Claim 17, wherein said cross-linking comprises exposing said surface to electromagnetic radiation.

20 19. In a method of performing a binding assay in which a solution suspected of comprising an analyte is contacted with a solid support bound biopolymeric binding member specific for said analyte and binding complexes between said analyte and support bound binding member are detected, the improvement comprising:

employing said array according to Claim 1 as said solid support bound binding agent.

25

20. A kit for use in performing a binding assay according to Claim 19, said kit comprising an array according to Claim 1.

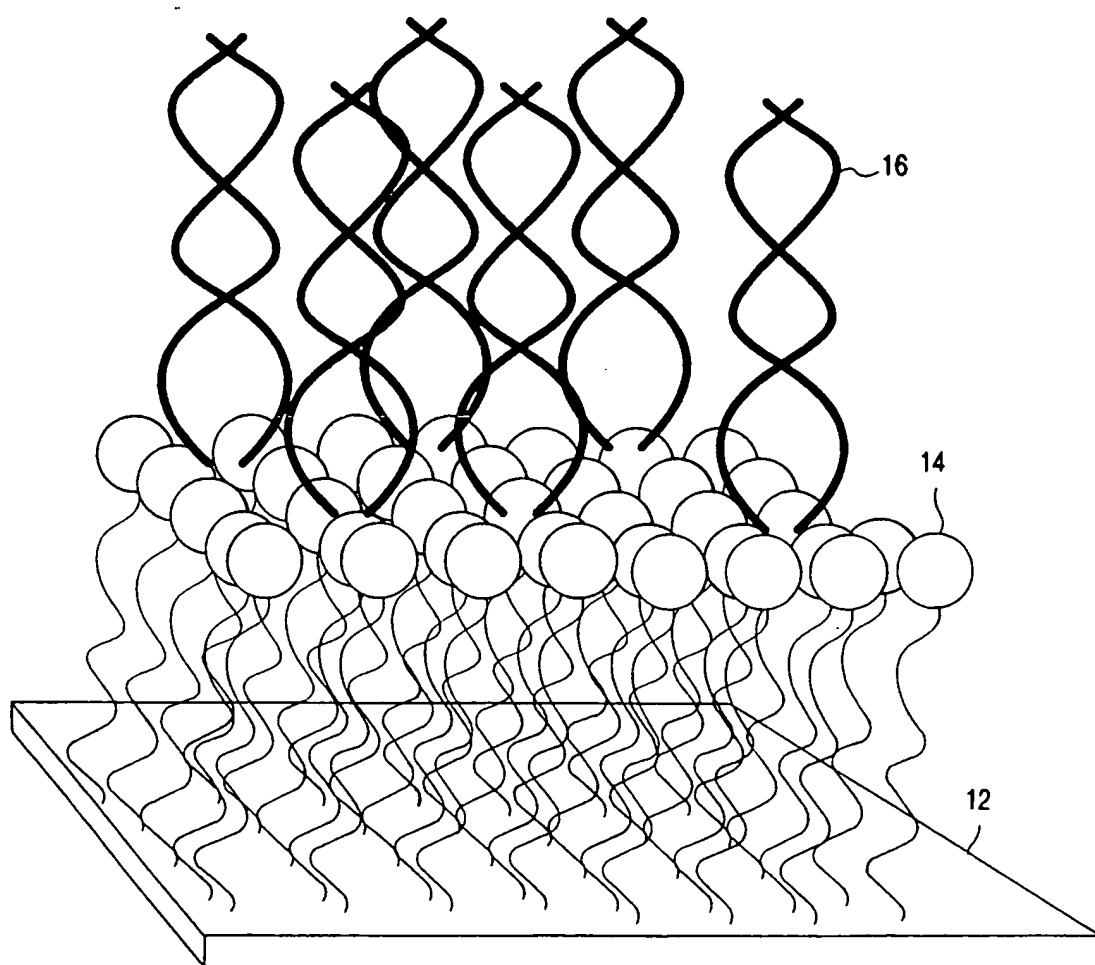


FIG. 1A



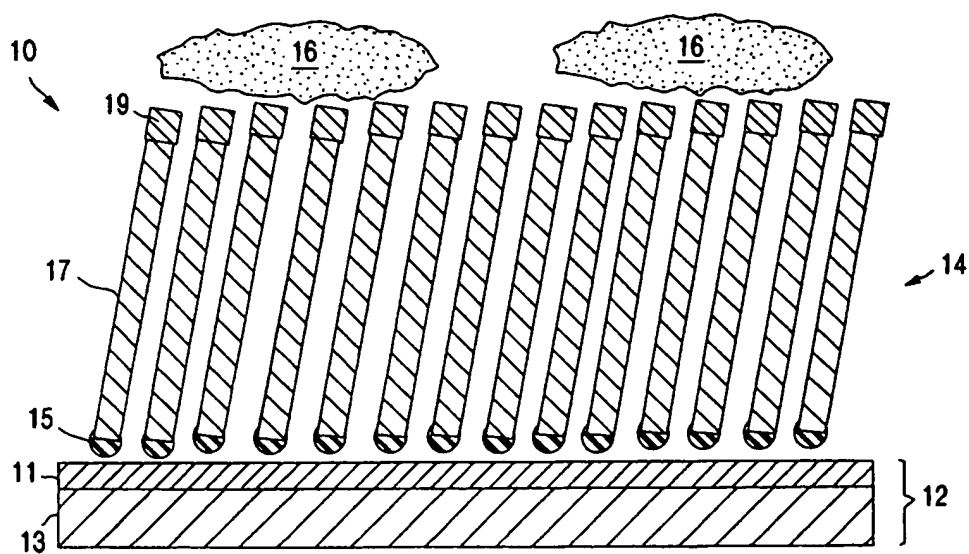
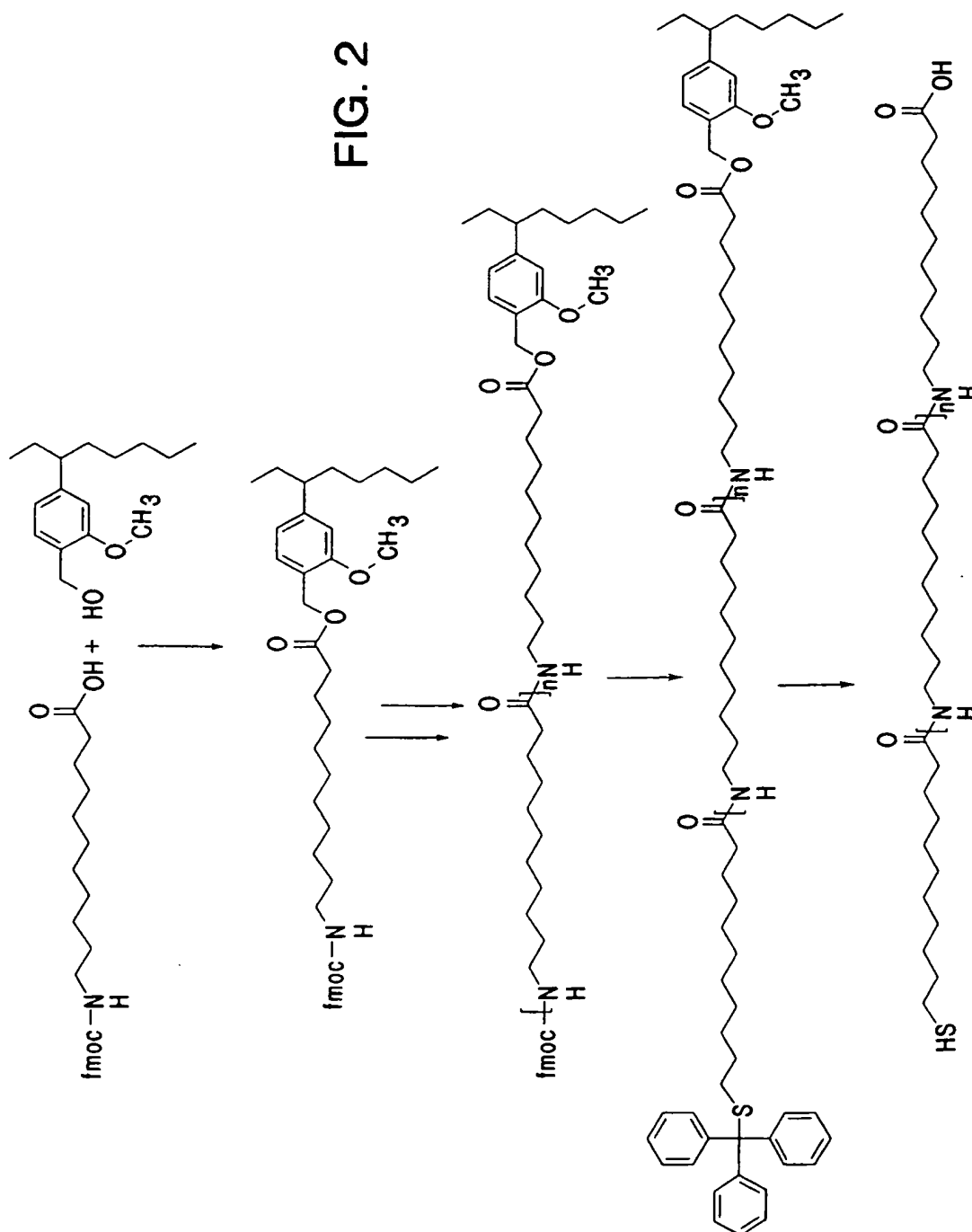


FIG. 1B

FIG. 2



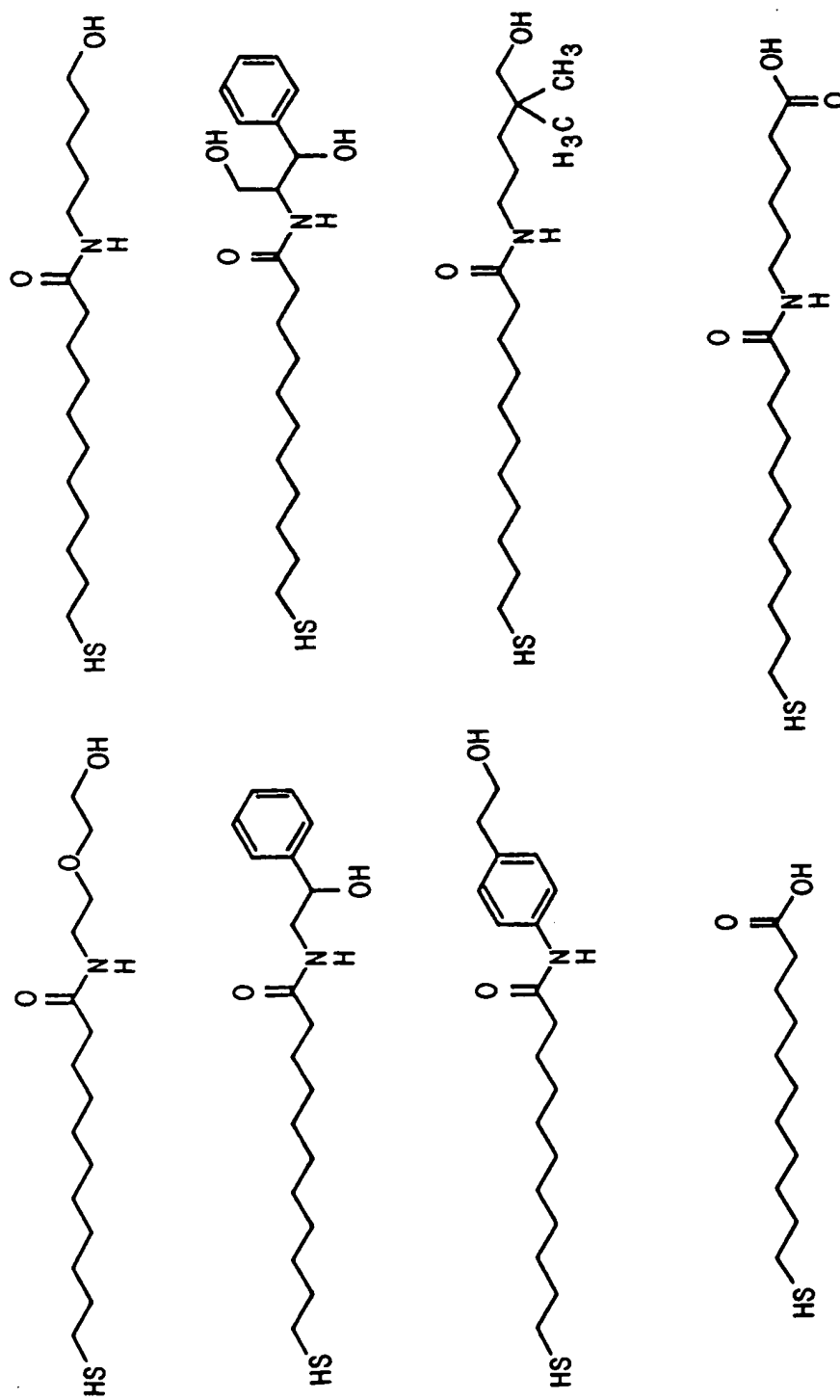
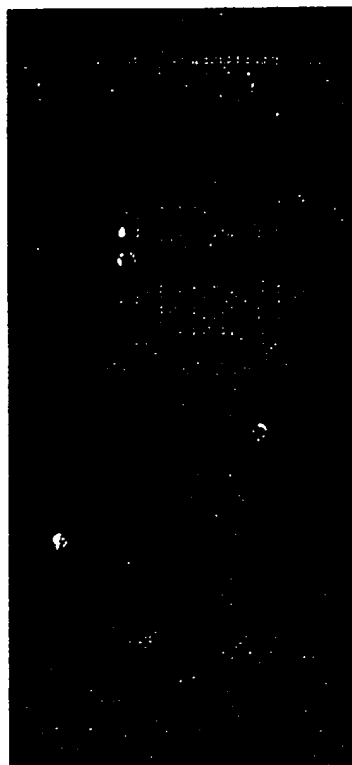


FIG. 3

5 / 5



Glass / APTES



Gold / MUA

- Cleaner / brighter background
- No negative spots
- 2-3 X signal enhancement
- Increased sensitivity - especially in Cy5

FIG. 4

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
4 January 2001 (04.01.2001)

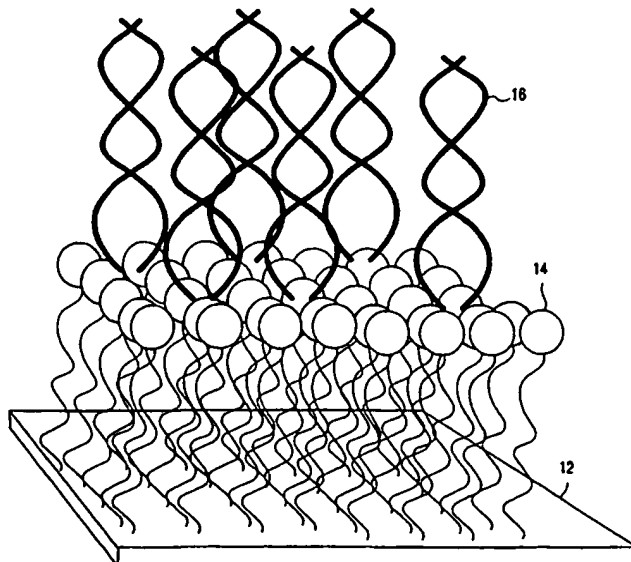
PCT

(10) International Publication Number  
**WO 01/01142 A3**

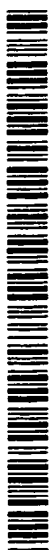
- (51) International Patent Classification<sup>7</sup>: G01N 33/543, C12Q 1/68
- (72) Inventor; and  
(75) Inventor/Applicant (*for US only*): CHARYCH, Deborah [US/US]; 909 Taylor Street, Albany, CA 94706 (US).
- (21) International Application Number: PCT/US00/16894
- (74) Agents: LENTINI, David, P. et al.; Chiron Corporation, Intellectual Property - R338, P.O. Box 8097, Emeryville, CA 94662-8097 (US).
- (22) International Filing Date: 19 June 2000 (19.06.2000)
- (25) Filing Language: English
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (26) Publication Language: English
- (30) Priority Data:  
60/141,469 29 June 1999 (29.06.1999) US
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- (63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:  
US 60/141,469 (CIP)  
Filed on 29 June 1999 (29.06.1999)
- (71) Applicant (*for all designated States except US*): CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608-2916 (US).
- Published:  
— with international search report

[Continued on next page]

(54) Title: ARRAYS OF BIOPOLYMERIC AGENTS AND METHOD FOR THEIR PRODUCTION AND USE



(57) Abstract: Arrays of biopolymeric binding agents, as well as methods for their fabrication and use, are provided. The subject arrays are characterized having at least two non-modified biopolymeric binding agents, e.g. proteins, nucleic acids, etc., bound to the hydrophilic surface of a spacer layer present a planar surface of a solid support, where the spacer layer at least includes a self-assembled monolayer. The subject arrays find use in a variety of different binding assay applications. Also provided are kits including the subject arrays.



WO 01/01142 A3

**WO 01/01142 A3**



**(88) Date of publication of the international search report:**  
30 August 2001

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

International Application No.

PCT/US 00/16894

**A. CLASSIFICATION OF SUBJECT MATTER**  
IPC 7 G01N33/543 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, EMBASE, CHEM ABS Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	THIEL A J ET AL: "IN SITU SURFACE PLASMON RESONANCE IMAGING DETECTION OF DNA HYBRIDIZATION TO OLIGONUCLEOTIDE ARRAYS ON GOLD SURFACES" ANALYTICAL CHEMISTRY, AMERICAN CHEMICAL SOCIETY, COLUMBUS, US, vol. 69, no. 24, 15 December 1997 (1997-12-15), pages 4948-4956, XP000733394 ISSN: 0003-2700 * see especially Fig.1 * the whole document --- -/--	1-20



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

\* Special categories of cited documents:

\*A\* document defining the general state of the art which is not considered to be of particular relevance

\*E\* earlier document but published on or after the international filing date

\*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

\*O\* document referring to an oral disclosure, use, exhibition or other means

\*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*G\* document member of the same patent family

Date of the actual completion of the international search

20 March 2001

Date of mailing of the international search report

27/03/2001

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Knehr, M

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BONCHEVA M ET AL.: "Design of oligonucleotide arrays at interfaces" LANGMUIR, vol. 15, 1999, pages 4317-4320, XP000991094 the whole document ---	1-20
Y	WO 98 31839 A (HARVARD COLLEGE) 23 July 1998 (1998-07-23) the whole document ---	1-17, 19, 20
Y	FOLKERS J P ET AL.: "Self-assembled monolayers of alkanethiols on gold: Comparisons of monolayers containing mixtures of short- and long-chain constituents with CH <sub>3</sub> and CH <sub>2</sub> OH terminal groups" LANGMUIR, vol. 8, 1992, pages 1330-1341, XP000979989 cited in the application abstract page 1340, column 2, paragraph 3 -page 1341, column 1, paragraph 1; figure 1 ---	1-17, 19, 20
Y	LEE M-T ET AL.: "Air oxidation of self-assembled monolayers on polycrystalline gold: The role of the gold substrate" LANGMUIR, vol. 14, 1998, pages 6419-6423, XP000979882 cited in the application the whole document ---	1-3, 6-9, 11, 12, 14-16
A	WO 93 22680 A (AFFYMAX TECH NV ; FODOR STEPHEN P A (US); MCGALL GLENN H (US); SHEL) 11 November 1993 (1993-11-11) the whole document ---	
A	WO 98 39481 A (ROGERS YU HUI ; ANDERSON STEPHEN (US); MOLECULAR TOOL INC (US)) 11 September 1998 (1998-09-11) the whole document ---	
P, X	FRUTOS A G ET AL.: "Reversible protection and reactive patterning of amine- and hydroxyl-terminated self-assembled monolayers on gold surfaces for the fabrication of biopolymer arrays" LANGMUIR, vol. 16, 2000, pages 2192-2197, XP000946578 the whole document --- -/--	1-20



C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 99 67425 A (CLINICAL MICRO SENSORS INC) 29 December 1999 (1999-12-29) * see especially example 1 * the whole document ----	1-20
P, X	BROCKMAN ET AL: "A Multistep Chemical Modification Procedure To Create DNA Arrays on Gold Surfaces for the Study of Protein-DNA Interactions with Surface Plasmon Resonance Imaging" JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, AMERICAN CHEMICAL SOCIETY, WASHINGTON, DC, US, vol. 121, no. 35, 1999, pages 8044-8051, XP002148625 ISSN: 0002-7863 the whole document ----	1-20
E	WO 00 67028 A (BROCKMAN JENNIFER M ; CORN ROBERT M (US); FRUTOS ANTHONY G (US); WI) 9 November 2000 (2000-11-09) the whole document -----	1-20

information on patent family members

International Application No.

PCT/US 00/16894

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9831839	A	23-07-1998	AU	5926598 A	07-08-1998
			EP	0981643 A	01-03-2000
WO 9322680	A	11-11-1993	US	5412087 A	02-05-1995
			AU	675054 B	23-01-1997
			AU	3148193 A	15-06-1993
			AU	4110793 A	29-11-1993
			CA	2124087 A	27-05-1993
			EP	0624059 A	17-11-1994
			EP	0916396 A	19-05-1999
			EP	0972564 A	19-01-2000
			JP	7506561 T	20-07-1995
			WO	9309668 A	27-05-1993
			US	6040193 A	21-03-2000
			US	6136269 A	24-10-2000
			US	5677195 A	14-10-1997
WO 9839481	A	11-09-1998	US	5837860 A	17-11-1998
			AU	6540298 A	22-09-1998
			EP	0910670 A	28-04-1999
			JP	2000510710 T	22-08-2000
			US	6030782 A	29-02-2000
WO 9967425	A	29-12-1999	AU	4709999 A	10-01-2000
WO 0067028	A	09-11-2000	US	6127129 A	03-10-2000
			AU	4640000 A	17-11-2000